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(54) Title: PROCESS FOR IDENTIFYING PARA CATION CHANNEL MODULATORS

(57) Abstract

DNAs encoding voltage-activated cation channels have been cloned and characterized. The cDNA's have been expressed in recombinant host cells which produce active recombinant protein. The recombinant protein is also purified from the recombinant host cells. In addition, the recombinant host cells are utilized to establish a method for identifying modulators of the channel activity, and channel modulators are identified. Channel modulators are useful as insecticides and arachnicidic agents.

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TITLE OF THE INVENTION

PROCESS FOR IDENTIFYING *PARA* CATION CHANNEL MODULATORS

BACKGROUND OF THE INVENTION

Voltage-activated sodium channels are responsible for the fast depolarizing phase of the action potential that underlies electrical signaling in neurons, muscles and other electrically excitable cells (reviewed by Hille, 1992 Ionic Channels of Excitable Membranes (Sinauer, Sunderland, MA)). Biochemical characterization of voltage-activated sodium channels from a variety of tissues indicate that they all contain a single alpha subunit of molecular weight ranging from 230,000 to 300,000 (reviewed by Catterall, 1992 Cellular and Molecular Biology of Voltage-gated Sodium Channels. *Physiological Reviews*, 72:S15-S48). The alpha subunit of the Electrophorus electricus voltage-activated sodium channel was cloned using biochemical and molecular genetic techniques (Noda, *et al.*, 1984 Primary structure of Electrophorus electricus sodium channel deduced from cDNA sequence. *Nature*, 312:121-127.). The purified Electrophorus electricus sodium channel alpha subunit forms a functional voltage-activated sodium channel as a single alpha subunit (Rosenberg, R.L., *et al.*, 1984, *Proc. Natn. Acad. Sci. U.S.A.* 81:1239-1243). The cDNA encoding the Electrophorus electricus voltage-activated sodium channel was used to isolate cDNAs encoding three distinct, but highly homologous rat brain voltage-activated sodium channel genes (Kayano *et al.*, 1988, Primary structure of rat brain sodium channel III deduced from the cDNA sequence, *FEBS Lett.* 228:187-194; Noda *et al.*, 1986, *Nature* 320:188-192). Biochemical analysis of voltage-activated sodium channels from rat brain indicate that the alpha subunits are associated noncovalently with a beta1 subunit (36,000 kDa) and are disulfide linked to a beta2 subunit (33,000 kDa) which is not required for channel activity (Hartshorne and Catterall, 1981, Purification of the saxitoxin receptor of the sodium channel from rat brain. *Proc. Natl. Acad. Sci. U.S.A.* 78:4620-4624; Hartshorne and

Catterall 1984, The sodium channel from rat brain. Purification and subunit composition. *J. Biol. Chem.* 259:1667-1675; Hartshorne, *et al.*, 1982, The saxitoxin receptor of the sodium channel from rat brain. Evidence for two nonidentical beta subunits. *J. Biol. Chem.* 257:13888-13891; Messsner and Catterall, 1985, The sodium channel from rat brain. Separation and characterization of subunits. *J. Biol. Chem.* 260:10597-10604). RNAs transcribed from cDNAs encoding alpha subunits of mammaliam voltage-activated sodium channels are sufficient to direct the synthesis of functional sodium channels when injected into Xenopus oocytes (Auld *et al.*, 1988, A rat brain Na+ channel alpha subunit with novel gating properties. *Neuron* 1:448-461; Moorman *et al.*, 1990, Changes in sodium channel gating produced by point mutations in a cytoplasmic linker. *Science* 250:688-691; Noda *et al.* 1986, Expression of functional sodium channels from cloned cDNA. *Nature* 322:826-828; Suzuki *et al.* 1988, Functional expression of cloned cDNA encoding sodium channel III. *FEBS Lett.* 228:195-200). Although alpha subunits of mammalian voltage-activated sodium channels are sufficient to encode functional sodium channels in Xenopus oocytes, their biophysical properties are not identical to those observed in intact cells. Co-expresssion of the rat brain voltage-activated sodium channel beta1 subunit with the rat brain type IIa alpha subunit in Xenopus oocytes restores the normal biophysical properties observed in intact cells (Isom *et al.* 1992, Primary structure and functional expression of the B1 subunit of the rat brain sodium channel. *Science* 256: 839-842).

Biochemical characterization of insect neuronal sodium channels has revealed that they contain an alpha subunit of molecular weight ranging from 240,000 to 280,000, but they lack any covalently linked beta subunits (Gordon *et al.* 1993, Biochemical Characterization of Insect Neuronal Sodium Channels. *Archives of Insect Biochemistry and Physiology* 22:41-53). Partial DNA sequences from the fruit fly Drosophila melanogaster presumed to encode voltage-activated sodium channels were initially identified on the basis of homology to vertebrate voltage-activated sodium channel alpha subunits (Salkoff *et al.* 1987,

Genomic organization and deduced amino acid sequence of a putative sodium channel genes in Drosophila. *Science* 237:744-749; Okamoto *et al.* 1987, Isolation of Drosophila genomic clones homologous to the eel sodium channel gene. *Proc. Jpn. Acad.* 63(B):284-288; Ramaswami and Tanouye, 1989, Two sodium-channel gene in Drosophila: Implications for channel diversity. *Proc. Natn. Acad. Sci. U.S.A.* 86:2079-2082). Using a molecular genetic approach it was determined that the paralytic (*para*) locus in Drosophila encodes a voltage-activated sodium channel alpha subunit and the entire *para* cDNA sequence was determined (Loughney *et al.* 1989, Molecular analysis of the *para* locus, a sodium channel gene in Drosophila. Cell 58:1143-1154; Thackeray and Ganetzky 1994, Developmentally regulated alternative splicing generates a complex array of Drosophila *para* sodium channel isoforms. *J. Neuroscience* 14:2569-2578).

It has been proposed that the Drosophila *tipE* locus encodes a regulatory or structural component of voltage-activated sodium channels for the following reasons: (1) [3H]saxitoxin binding to voltage-activated sodium channels is reduce 30-40% in *tipE* mutants (Jackson *et al.* 1986, The *tipE* mutation of Drosophila decreases saxitoxin binding and interacts with other mutations affecting nerve membrane excitability. *J. of Neurogenetics*, 3:1-17), (2) sodium current density is reduced 40-50% in cultured embryonic neurons from *tipE* mutants (O'Dowd and Aldrich, 1988, Voltage-Clamp Analysis of Sodium Channels in wild-type and Mutant Drosophila *Neurons. J. of Neuroscience*, 8:3633-3643), (3) *para;tipE* mutants exhibit unconditional lethality in an allele specific manner (Ganetzky 1986, Neurogenetic analysis of Drosophila Mutations affecting Sodium Channels: Synergistic Effects on Viability and Nerve Conduction in Double Mutants involving *tipE*. *J. of Neurogenetics*, 3:19-31; Jackson *et al.* 1986, The *tipE* mutation of Drosophila decreases saxitoxin binding and interacts with other mutations affecting nerve membrane excitability. *J. of Neurogenetics*, 3:1-17), (4) *para* and *tipE* RNA are expressed in the embryonic CNS and PNS (Hall *et al.* 1994, Molecular and genetic analysis of *tipE*: a mutation affecting sodium channels in

Drosophila. Presented at the 35th Annual Drosophila Research Conference, April 20-24, 1994, Chicago, Illinois; Hong and Ganetzky 1994, Spatial and temporal expression patterns of two sodium channel genes in Drosophila. *J. Neuroscience*, 14:5160-5169), (5) *tipE* encodes a 50kDa acidic protein with two putative membrane spanning domains, a membrane topology shared by other ion channel subunits (Hall *et al.* 1994, Molecular and genetic analysis of *tipE*: a mutation affecting sodium channels in Drosophila. Presented at the 35th Annual Drosophila Research Conference, April 20-24, 1994, Chicago, Illinois; Hall and Feng 1994, The *tipE* locus defines a novel membrane protein required during development to rescue adult *para*lysis. Presented at the 48th annual meeting of the Society of General Physiologists, September 7-11, 1994, Woods Hole Massachuetts). The Drosophila *tipE* locus has been cloned and sequenced but the nucleotide and amino acid sequence of *tipE* are presently undisclosed (Hall *et al.* 1994, Molecular and genetic analysis of *tipE*: a mutation affecting sodium channels in Drosophila. Presented at the 35th Annual Drosophila Research Conference, April 20-24, 1994, Chicago, Illinois; Hall and Feng 1994, The *tipE* locus defines a novel membrane protein required during development to rescue adult paralysis (*para*). Presented at the 48th annual meeting of the Society of General Physiologists, September 7-11, 1994, Woods Hole Massachuetts).

SUMMARY OF THE INVENTION

Using a recombinant expression system, it has been shown that functional expression of Drosophila *para* voltage-activated sodium channels requires the co-expression of the *para* alpha subunit with *tipE*, a putative Drosophila voltage-activated sodium channel beta subunit. The electrophysiological and pharmachological properties of the Drosophila *para* voltage-activated sodium channel is disclosed. Recombinant host cells expressing the Drosophila *para* voltage-activated sodium channel are useful in the identification of modulators of insect voltage-activated sodium channels. Modulators of voltage-activated sodium channels are useful as insecticides and therapeutic agents.

Voltage-activated sodium channel *para* homologs from other arthropod species are likely to also require coexpression with the corresponding *tipE* homolog for functional expression.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 - PCR amplification and assemble of a full length *para* cDNA is shown.

Figure 2 - Construction of a functional full length *para* cDNA is shown.

Figure 3 Panels A, B, and C - Expression of tetrodotoxin-sensitive sodium currents in Xenopus oocytes injected with *para* and *tipE* mRNA produced by *in vitro* transcription is shown.

Figure 4 - Steady-state voltage dependence of inactivation for *para* sodium currents is shown.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to coexpression of *para* and *tipE* cDNAs encoding a Drosophila voltage-activated sodium channel. The present invention is also related to recombinant host cells which coexpress the cloned *para* and *tipE* encoding DNAs contained in recombinant expression plasmids. The present invention is also related to a method for the screening of substances which modulate Drosophila *para* voltage-activated sodium channel activity. The amino acid sequence of *para* and the DNA encoding *para* were previously known (Loughney *et al*. 1989, Molecular analysis of the *para* locus, a sodium channel gene in Drosophila. *Cell* 58:1143-1154; Thackeray and Ganetzky 1994, Developmentally Regulated alternative splicing generates a complex array of Drosophila *para* sodium channel isoforms. *J. Neuroscience* 14:2569-2578) and PCR generated full length *para* cDNA clones are described herein (see Figure 1)

Partial DNA sequences from the insect, Drosophila melanogaster presumed to encode voltage-activated sodium channels were initially identified on the basis of homology to vertebrate voltage-activated sodium channel alpha subunits (Salkoff *et al.* 1987, Genomic organization and deduced amino acid sequence of a putative sodium channel genes in Drosophila. *Science* 237:744-749; Okamoto *et al.* 1987, Isolation of Drosophila genomic Clones homologous to the eel sodium channel gene. *Proc. Jpn. Acad.* 63(B):284-288; Ramaswami and Tanouye, 1989, Two sodium-channel gene in Drosophila: Implications for channel diversity. *Proc. Natn. Acad. Sci. U.S.A.* 86:2079-2082). Using a molecular genetic approach it was determined that the *para* locus in Drosophila encodes a voltage-activated sodium channel alpha subunit and the entire *para* cDNA sequence was determined from a series of overlapping cDNA clones (Loughney *et al.* 1989, *supra*, Thackeray and Ganetzky 1994, *supra*). It is readily apparent to those skilled in the art that a number of approaches could be used to assemble a full length *para* cDNA for functional expression studies. These methods include, but are not limited to, assembling the availiable partial cDNAs into a full length cDNA, using the exisiting cDNA clones to screen a Drosophila cDNA library to isolate a full length cDNA, PCR amplification of a full length cDNA using primers based on the published sequence. The actual method employed for the invention described herein is summarized in Figure 1 and Figure 2.

It is readily apparent to those skilled in the art that suitable cDNA libraries may be prepared from tissue derived from any developmental stage of Drosophila which have voltage-activated sodium channel activity or any Drosophila cell line exhibiting voltage-activated sodium channel activity. The selection of tissues or cell lines for use in preparing a cDNA library to isolate *para* cDNA may be done by first measuring *para* expression using the known *para* DNA sequence or available *para* cDNAs to generate a probe.

Preparation of cDNA libraries and analysis of *para* expression can be performed by standard techniques well known in the art. Well known cDNA library construction techniques and RNA

analysis techniques can be found for example, in Maniatis, T., Fritsch, E.F., Sambrook, J., Molecular Cloning: A Laboratory Manual (Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1982). Well know techniques for PCR amplification of DNA and RNA can be found for example, in Innis, M.A., Gelfand, D.H., Sninsky, J.J., White, T.J., PCR Protocols: A Guide to Methods and Applications (Academic Press, Inc., San Diego, California, 1990).

The nucleotide and deduced amino acid sequence of *tipE* are presently undisclosed; however, the DNA encoding *tipE* has been cloned and sequenced (Hall *et al.* 1994, Molecular and genetic analysis of *tipE*: a mutation affecting sodium channels in Drosophila. Presented at the 35th Annual Drosophila Research Conference, April 20-24, 1994, Chicago, Illinois; Hall and Feng 1994, The *tipE* locus defines a novel membrane protein required during development to rescue adult *para*lysis. Presented at the 48th annual meeting of the Society of General Physiologists, September 7-11, 1994, Woods Hole Massachuetts) and was used to provide *tipE* RNA for use herein.

It is readily apparent to those skilled in the art that a number of approaches can be used to clone the Drosophila *tipE* locus. These methods include, but are not limited to, chromosome walking to identify chromosomal rearrangements associated with a *tipE* mutation followed by isolating a cDNA corresponding to the transcription unit disrupted by the chromosomal rearrangement (as described by Hall *et al.* 1994, *supra*). Another method is to generate *tipE* mutations with transposable element insertions followed by cloning of the DNA flanking the transposible element insertion and using this DNA to screen a Drosophila head specific cDNA library which is enriched in clones derived from neuronal RNAs.

Cloning of Drosophila genes can be performed by standard techniques well know in the art. Well known Drosophila molecular genetic techniques can be found for example, in Roberts, D.B., Drosophila A Practical Approach (IRL Press, Washington, D.C., 1986). Preparation of cDNA libraries can be performed by standard techniques well known in the art. Well known cDNA library construction

techniques can be found for example, in Maniatis, T., Fritsch, E.F., Sambrook, J., Molecular Cloning: A Laboratory Manual (Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1982).

Purified biologically active *para* voltage-activated sodium channels may have several different physical forms. *Para* and *tipE* may exist as a full-length nascent or unprocessed polypeptide, or as partially processed polypeptides or combinations of processed polypeptides. *Para* and/or *tipE* may be encoded by differentially spliced RNAs leading to different *para* and/or *tipE* protein isoforms with different primary amino acid sequences. The full-length nascent *para* and/or *tipE* polypeptide may be postranslationally modified by specific proteolytic cleavage events which result in the formation of fragments of the full length nascent polypeptide. A fragment, or physical association of fragments may have the full biological activity associated with *para* and *tipE* (voltage-activated sodium channel) however, the degree of sodium channel activity may vary between individual *para* and *tipE* fragments and physically associated *para* and *tipE* polypeptide fragments.

Biologically active *para* voltage-activated cation channels may be encoded by a variety of alternatively spliced mRNA. Expression of the alternatively spliced *para* mRNA may result in different biologically active isoforms of the *para* channel (Thackeray and Ganetzky, 1994, *supra*). These isoforms of *para* may not require the *tipE* subunit for biological activity. Various isoforms of *para* are intended to be encompassed by the present invention provided that the *para* isoform has the biological activity described herein. In addition, biologically active *para* voltage-activated sodium channels may have several different physical forms. The active *para* voltage-activated sodium channel may exist as a complex containing both *para* and *tipE* polypeptides, or the active *para* voltage-activated sodium channel may consist of *para* alone.

The cloned *para* and *tipE* cDNAs obtained through the methods described above may be recombinantly expressed by molecular cloning into an expression vector containing a suitable promoter and other appropriate transcription regulatory elements, and transferred

into prokaryotic or eukaryotic host cells to produce recombinant *para* and *tipE*. Techniques for such manipulations can be found described in Maniatis, T, *et al.*, *supra*, and are well known in the art.

Expression vectors are defined herein as DNA sequences that are required for the transcription of cloned DNA and the translation of their mRNAs in an appropriate host. Such vectors can be used to express eukaryotic DNA in a variety of hosts such as bacteria, bluegreen algae, fungal cells, plant cells, insect cells and animal cells.

Specifically designed vectors allow the shuttling of DNA between hosts such as bacteria-yeast or bacteria-animal cells. An appropriately constructed expression vector should contain: an origin of replication for autonomous replication in host cells, selectable markers, a limited number of useful restriction enzyme sites, a potential for high copy number, and active promoters. A promoter is defined as a DNA sequence that directs RNA polymerase to bind to DNA and initiate RNA synthesis. A strong promoter is one which causes mRNAs to be initiated at high frequency. Expression vectors may include, but are not limited to, cloning vectors, modified cloning vectors, specifically designed plasmids or viruses.

A variety of mammalian expression vectors may be used to express recombinant *para* and *tipE* in mammalian cells. Commercially available mammalian expression vectors which may be suitable for recombinant *para* and *tipE* expression, include but are not limited to, pMAMneo (Clontech), pMClneo, pXT1, pSG5 (Stratagene), pcDNAI, pcDNAIamp, pcDNA3 (Invitrogen), EBO-pSV2-neo (ATCC 37593) pBPV-1(8-2) (ATCC 37110), pdBPV-MMTneo(342-12) (ATCC 37224), pRSVgpt (ATCC 37199), pRSVneo (ATCC 37198), pSV2-dhfr (ATCC 37146), pUCTag (ATCC 37460), and lZD35 (ATCC 37565)

A variety of bacterial expression vectors may be used to express recombinant *para* and *tipE* in bacterial cells. Commercially available bacterial expression vectors which may be suitable for recombinant expression include, but are not limited to, pET vectors (Novagen) and pQE vectors (Qiagen).

A variety of fungal cell expression vectors may be used to express recombinant *para* and *tipE* in fungal cells such as yeast. Commerically available fungal cell expression vectors which may be suitable for recombinant expression include, but are not limited to, pYES2 (Invitrogen) and Pichia expression vector (Invitrogen).

A variety of insect cell expression vectors may be used to express recombinant *para* and *tipE* in insect cells. Commercially available insect cell expression vectors which may be suitable for recombinant expression include, but are not limited to, pBlueBacII (Invitrogen).

DNA encoding *para* and *tipE* may also be cloned into an expression vector for expression in a recombinant host cell. Recombinant host cells may be prokaryotic or eukaryotic, including but not limited to bacteria such as *E. coli*, fungal cells such as yeast, mammalian cells including but not limited to cell lines of human, bovine, porcine, monkey and rodent origin, and insect cells including but not limited to Drosophila (Schneider-2, Kc, etc.) and silkworm derived cell lines. Cell lines derived from mammalian species which may be suitable and which are commercially available, include but are not limited to, CV-1 (ATCC CCL 70), COS-1 (ATCC CRL 1650), COS-7 (ATCC CRL 1651), CHO-K1 (ATCC CCL 61), 3T3 (ATCC CCL 92), NIH/3T3 (ATCC CRL 1658), HeLa (ATCC CCL 2), C127I (ATCC CRL 1616), BS-C-1 (ATCC CCL 26), MRC-5 (ATCC CCL 171), L-cells, and HEK-293 (ATCC CRL1573).

The expression vector may be introduced into host cells via any one of a number of techniques including but not limited to transformation, transfection, protoplast fusion, lipofection, and electroporation. The expression vector-containing cells are clonally propagated and individually analyzed to determine whether they produce *para* and *tipE* protein. Identification of *para* and *tipE* expressing host cell clones may be done by several means, including but not limited to immunological reactivity with anti-*para* or anti-*tipE* antibodies, and the presence of host cell-associated voltage-activated sodium channel activity.

Expression of *para* and *tipE* DNA may also be performed using *in vitro* produced synthetic mRNA. Synthetic mRNA or mRNA isolated from *para* voltage-activated sodium channel producing cells can be efficiently translated in various cell-free systems, including but not limited to wheat germ extracts and reticulocyte extracts, as well as efficiently translated in cell based systems, including but not limited to microinjection into frog oocytes, with microinjection into frog oocytes being preferred.

While functional expression of the *para* cation channel in Xenopus oocytes required the coexpression of *tipE*, other expression systems in other recombinant host cells may not require coexpression with *tipE*. Such alternate expression systems and host cells include, but are not limited to, mammalian cells, insect cells, fungal cells, and bacterial cells.

To determine the *para* and *tipE* DNA sequence(s) that yields optimal levels of voltage-activated sodium channel activity and/or sodium channel protein, *para* and *tipE* DNA molecules including, but not limited to, the following can be constructed: the full-length open reading frame of the *para* and *tipE* cDNA and various constructs containing portions of the cDNA encoding only specific domains of the ion channel proteins or rearranged domains of the proteins, or alternative splice forms of *para* or *tipE*. All constructs can be designed to contain none, all or portions of the 5' and/or 3' untranslated region of the *para* and/or *tipE* cDNAs. Voltage-activated sodium channel activity and levels of protein expression can be determined following the introduction, both singly and in combination, of these constructs into appropriate host cells. Following determination of the *para* and *tipE* cDNA cassettes yielding optimal expression in transient assays, these *para* and *tipE* cDNA constructs are transferred to a variety of expression vectors (including recombinant viruses), including but not limited to those for mammalian cells, plant cells, insect cells, oocytes, baculovirus-infected insect cells, *E. coli*, and the yeast *S. cerevisiae*.

Host cell transfectants and microinjected oocytes may be assayed for both the levels of voltage-activated sodium channel activity

and levels of *para* and *tipE* protein by the following methods. In the case of recombinant host cells, this involves the co-transfection of one or possibly two or more plasmids, containing the *para* and *tipE* DNA. In the case of oocytes, this involves the co-injection of synthetic RNAs for *para* and *tipE*. Following an appropriate period of time to allow for expression, cellular protein is metabolically labelled with for example ^{35}S-methionine for 24 hours, after which cell lysates and cell culture supernatants are harvested and subjected to immunprecipitation with polyclonal antibodies directed against the *para* and/or *tipE* proteins.

Other methods for detecting *para* activity involve the direct measurement of voltage-activated sodium channel activity in whole or fractionated cells transfected with *para* and *tipE* cDNA or oocytes injected with *para* and *tipE* mRNA. Voltage-activated sodium channel activity is measured by membrane depolarization and electrophysiological characteristics of the host cells expressing *para* and *tipE* DNA. In the case of recombinant host cells expressing *para* and *tipE*, patch voltage clamp techniques can be used to measure sodium channel activity and quantitate *para* and *tipE* protein. In the case of oocytes patch clamp as well as two electrode voltage clamp techniques can be used to measure sodium channel activity and quantitate *para* and *tipE* protein.

Levels of *para* and *tipE* protein in host cells are quantitated by immunoaffinity and/or ligand affinity techniques. Cells expressing *para* and *tipE* can be assayed for the number of *para* molecules expressed by measuring the amount of radioactive saxitoxin binding to cell membranes. *para*- or *tipE*-specific affinity beads or *para*- or *tipE*-specific antibodies are used to isolate for example ^{35}S-methionine labelled or unlabelled sodium channel proteins. Labelled *para* and *tipE* proteins are analyzed by SDS-PAGE. Unlabelled *para* and *tipE* proteins are detected by Western blotting, ELISA or RIA assays employing *para* or *tipE* specific antibodies.

Following expression of *para* and *tipE* in a recombinant host cell, *para* and *tipE* protein may be recovered to provide *para* sodium channels in active form. Several *para* sodium channel

purification procedures are available and suitable for use. As described herein for purification of *para* from natural sources, recombinant *para* may be purified from cell lysates and extracts, or from conditioned culture medium, by various combinations of, or individual application of salt fractionation, ion exchange chromatography, size exclusion chromatography, hydroxylapatite adsorption chromatography and hydrophobic interaction chromatography.

In addition, recombinant *para* can be separated from other cellular proteins by use of an immunoaffinity column made with monoclonal or polyclonal antibodies specific for full length nascent *para*, polypeptide fragments of *para* or *para* subunits.

Monospecific antibodies to *para* or *tipE* are purified from mammalian antisera containing antibodies reactive against *para* or *tipE* or are prepared as monoclonal antibodies reactive with *para* or *tipE* using the technique of Kohler and Milstein, *Nature* 256: 495-497 (1975). Monospecific antibody as used herein is defined as a single antibody species or multiple antibody species with homogenous binding characteristics for *para* or *tipE*. Homogenous binding as used herein refers to the ability of the antibody species to bind to a specific antigen or epitope, such as those associated with the *para* or *tipE*, as described above. *Para* or *tipE* specific antibodies are raised by immunizing animals such as mice, rats, guinea pigs, rabbits, goats, horses and the like, with rabbits being preferred, with an appropriate concentration of *para* or *tipE* either with or without an immune adjuvant.

Preimmune serum is collected prior to the first immunization. Each animal receives between about 0.1 mg and about 1000 mg of *para* or *tipE* associated with an acceptable immune adjuvant. Such acceptable adjuvants include, but are not limited to, Freund's complete, Freund's incomplete, alum-precipitate, water in oil emulsion containing Corynebacterium parvum and tRNA. The initial immunization consists of *para* or *tipE* in, preferably, Freund's complete adjuvant at multiple sites either subcutaneously (SC), intraperitoneally (IP) or both. Each animal is bled at regular intervals, preferably weekly, to determine antibody titer. The animals may or may not

receive booster injections following the initial immunization. Those animals receiving booster injections are generally given an equal amount of the antigen in Freund's incomplete adjuvant by the same route. Booster injections are given at about three week intervals until maximal titers are obtained. At about 7 days after each booster immunization or about weekly after a single immunization, the animals are bled, the serum collected, and aliquots are stored at about -20°C.

Monoclonal antibodies (mAb) reactive with *para* or *tipE* are prepared by immunizing inbred mice, preferably Balb/c, with *para* or *tipE*. The mice are immunized by the IP or SC route with about 0.1 mg to about 10 mg, preferably about 1 mg, of *para* or *tipE* in about 0.5 ml buffer or saline incorporated in an equal volume of an acceptable adjuvant, as discussed above. Freund's complete adjuvant is preferred. The mice receive an initial immunization on day 0 and are rested for about 3 to about 30 weeks. Immunized mice are given one or more booster immunizations of about 0.1 to about 10 mg of *para* in a buffer solution such as phosphate buffered saline by the intravenous (IV) route. Lymphocytes, from antibody positive mice, preferably splenic lymphocytes, are obtained by removing spleens from immunized mice by standard procedures known in the art. Hybridoma cells are produced by mixing the splenic lymphocytes with an appropriate fusion partner, preferably myeloma cells, under conditions which will allow the formation of stable hybridomas. Fusion partners may include, but are not limited to: mouse myelomas P3/NS1/Ag 4-1; MPC-11; S-194 and Sp 2/0, with Sp 2/0 being preferred. The antibody producing cells and myeloma cells are fused in polyethylene glycol, about 1000 mol. wt., at concentrations from about 30% to about 50%. Fused hybridoma cells are selected by growth in hypoxanthine, thymidine and aminopterin supplemented Dulbecco's Modified Eagles Medium (DMEM) by procedures known in the art. Supernatant fluids are collected from growth positive wells on about days 14, 18, and 21 and are screened for antibody production by an immunoassay such as solid phase immunoradioassay (SPIRA) using *para* or *tipE* as the antigen. The culture fluids are also tested in the Ouchterlony precipitation assay

to determine the isotype of the mAb. Hybridoma cells from antibody positive wells are cloned by a technique such as the soft agar technique of MacPherson, Soft Agar Techniques, in Tissue Culture Methods and Applications, Kruse and Paterson, Eds., Academic Press, 1973.

Monoclonal antibodies are produced *in vivo* by injection of pristane primed Balb/c mice, approximately 0.5 ml per mouse, with about 2 x 10^6 to about 6 x 10^6 hybridoma cells about 4 days after priming. Ascites fluid is collected at approximately 8-12 days after cell transfer and the monoclonal antibodies are purified by techniques known in the art.

In vitro production of anti-*para* or anti-*tipE* mAb is carried out by growing the hydridoma in DMEM containing about 2% fetal calf serum to obtain sufficient quantities of the specific mAb. The mAb are purified by techniques known in the art.

Antibody titers of ascites or hybridoma culture fluids are determined by various serological or immunological assays which include, but are not limited to, precipitation, passive agglutination, enzyme-linked immunosorbent antibody (ELISA) technique and radioimmunoassay (RIA) techniques. Similar assays are used to detect the presence of *para* or *tipE* in body fluids or tissue and cell extracts.

It is readily apparent to those skilled in the art that the above described methods for producing monospecific antibodies may be utilized to produce antibodies specific for *para* or *tipE* polypeptide fragments, or full-length nascent *para* or *tipE* polypeptide, or the individual *para* or *tipE* subunits. Specifically, it is readily apparent to those skilled in the art that monospecific antibodies may be generated which are specific for only *para* or *tipE* or the fully functional voltage-activated sodium channel.

Para and *tipE* antibody affinity columns are made by adding the antibodies to Affigel-10 (Biorad), a gel support which is activated with N-hydroxysuccinimide esters such that the antibodies form covalent linkages with the agarose gel bead support. The antibodies are then coupled to the gel via amide bonds with the spacer arm. The remaining activated esters are then quenched with 1M

ethanolamine HCl (pH 8). The column is washed with water followed by 0.23 M glycine HCl (pH 2.6) to remove any non-conjugated antibody or extraneous protein. The column is then equilibrated in phosphate buffered saline (pH 7.3) and the cell culture supernatants or cell extracts containing *para* and *tipE* or only one subunit are slowly passed through the column. The column is then washed with phosphate buffered saline until the optical density (A280) falls to background, then the protein is eluted with 0.23 M glycine-HCl (pH 2.6). The purified *para* or *tipE* protein is then dialyzed against phosphate buffered saline.

When coexpressed in Xenopus oocytes *para* and *tipE* encode proteins that produce a voltage-activated sodium channel that is blocked by tetrodotoxin. The novel Drosophila voltage-activated sodium channel of the present invention is suitable for use in an assay procedure for the identification of compounds which modulate sodium channel activity. Modulating sodium channel activity, as described herein includes the inhibition or activation of the channel and also includes directly or indirectly affecting the normal regulation of the sodium channel activity. Compounds which modulate the sodium channel activity include agonists, antagonists and compounds which directly or indirectly affect regulation of the sodium channel activity.

The Drosophila voltage-activated sodium channel of the present invention may be obtained from both native and recombinant sources for use in an assay procedure to identify receptor modulators. In general, an assay procedure to identify insect sodium channel modulators will contain the *para* voltage-activated sodium channel of the present invention, and a test compound or sample which contains a putative sodium channel modulator. The test compounds or samples may be tested directly on, for example, purified sodium channel protein whether native or recombinant, subcellular fractions of sodium channel-producing cells whether native or recombinant, and/or whole cells expressing the sodium channel whether native or recombinant. The test compound or sample may be added to the sodium channel in the presence or absence of a known labelled or unlabelled sodium channel modulator. The modulating activity of the test compound or sample

may be determined by, for example, analyzing the ability of the test compound or sample to bind to the sodium channel, activate the sodium channel, inhibit sodium channel activity, inhibit or enhance the binding of other compounds to the sodium channel, modify sodium channel regulation, modify an intracellular activity, or kill the cell expressing the sodium channel.

It is likely that *para* and *tipE* related genes in other arthropods encode subunits of voltage-activated sodium channels and that functional expression of the homologous *para* sodium channel in these species will also require co-expression with the homologous *tipE* subunit. *Para* homologs have been partially cloned and characterized in the house fly, Musca domestica, (Williamson *et al*. 1993, Knockdown resistance (kdr) to DDT and pyrethroid insectcides maps to a sodium channel gene locus in the housefly (Musca domestica). *Mol Gen Genet* 240:17-22; Knipple *et al*., 1994, Tight genetic linkage between the kdr insecticide resistance trait and a voltage-sensitive sodium channel gene in the house fly. *Proc. Natn. Acad. Sci. U.S.A.* 91:2483-2487) and in the tobacco budworm, Heliothis virescens (Taylor *et al*. 1993, Linkage of pyrethroid insecticide resistance to a sodium channel locus in the tobacco budworm. *Insect Biochem. Molec. Biol*. 23:763-775); these *para* homologs share 92% and 89% identity to the Drosophila melanogaster *para* gene, respectively. The high degree of amino acid identity shared by these *para* homologs may be indicative of the structural and functional conservation of *para* sodium channels between insects. Furthermore, resistance to pyrethroid insecticides maps to the *para* locus in all three species (Hall, L. and Kasbekar, D, 1989, in: *Insecticide Action*, pp. 99-114, Narahashi and Chambers (eds.), Plenum Press, New York; Williamson *et al., supra*; Knipple *et al., supra*; Taylor *et al., supra*); therefore, it is likely that functional expression of all insect *para* voltage-activated sodium channels will require co-expression with *tipE*.

The identification of modulators of *para* sodium channel activity are useful as insecticides and arachnicides. Selective modulators, antagonists or agonists of the insect sodium channel may be

used to combat agricultural pests which attack crops either in the field or in storage, pests that attack forestry stock, insect pest infestations in general, nematodes, or fungi which infect plants and/or animals. The coumpounds are applied for such uses as sprays, dusts, emulsions and the like either to the growing plants or the harvested crops. The techniques for applying these compounds in this manner are known to those skilled in the agricultural arts. Selective modulators, antagonists or agonists of the insect sodium channel may also be used in the prevention and treatment of parasitic infections in humans and domestic animals by ectoparasites such as ticks, mites, lice, fleas and the like. The techniques for administering these compounds to animals and humans are known to those skilled in the veterinary and human health fields, respectively. Other compounds may be useful for stimulating or inhibiting the activity of the sodium channels. Selective antagonists of human sodium channels may be useful as neuro-protetive agents for the treatment of stroke, head injury and other ischemic events.

The following examples are provided for the purpose of illustrating the present invention without, however, limiting the same thereto.

EXAMPLE 1

Cloning of a full length *para* cDNA

A series of full length *para* cDNA clones were obtained by PCR amplification of three overlapping regions of the *para* cDNA followed by assembly of a composite full length clone as outlined in figure 1. A detailed description of the scheme used follows. Attempts to amplify the entire 6500 bp *para* cDNA in a single PCR reaction were unsuccessful; therefore, a number of *para* cDNAs were generated from a series of three overlapping PCR generated fragments (Figure 1). Oligonucleotide primers were designed based on the known *para* cDNA sequence (Loughney *et al.* 1989, Molecular analysis of the *para* locus, a sodium channel gene in Drosophila. *Cell* 58:1143-1154; Thackeray and

Ganetzky 1994, Developmentally Regulated alternative splicing generates a complex array of Drosophila *para* sodium channel isoforms. *J. Neuroscience* 14:2569-2578) and the primer sequences were primer 1- GACTCTAGACGTTGGCCGCATAGACAATGACAG [SEQ.ID.NO.:1], primer 2- AAGAGCTCGACGAAGGGATCG [SEQ.ID.NO.:2], primer 3- TCTTCGATCCCTTCGTCGAGCTCT [SEQ.ID.NO.:3], primer 4- AAAGGATCCAAATATGATGAA [SEQ.ID.NO.:4], primer 5- TTTGGATCCTTTTTCACACTCAATC [SEQ.ID.NO.:5], primer 6- GACTCTAGAGCTAATACTCGCGTGC-ATCTTGG [SEQ.ID.NO.:6]. A number of independent PCR generated *para* cDNA fragments for each segment were isolated and subcloned into the pBluescript SK(+) vector (Stratagene). These *para* cDNA fragments were assembled into five different full length *para* cDNA clones with different combinations of alternative exons in the first two fragments, but the 3' fragment of each clone was identical.

Sequence analysis of the PCR generated cDNA clones revealed that they contained a number of PCR induced nucleotide substitutions resulting in alteration and truncation of the encoded *para* protein; and therefore, these cDNA clones could not be used for functional expression. A cDNA clone suitable for functional expression was constructed by combining existing PCR generated cDNA clones, an existing cDNA clone isolated from a Drosophila head specific cDNA library (Loughney *et al.* 1989, *supra*) and new PCR generated cDNA clones as outlined in figure 2. The nucleotide sequence of the *para* cDNA insert in pGH19-13-5 was determined to confirm that it encoded a full length *para* protein.

A 6513 bp composite *para* cDNA clone used for functional expression has the following nucleotide sequence:

TCTAGACGTTGGCCGCATAGACAATGACAGAAGATTCCGACTCGATATCT
GAGGAAGAACGCAGTTTGTTCCGTCCCTTTACCCGCGAATCATTGGTGCA
AATCGAACAACGCATTGCCGCTGAACATGAAAAGCAGAAGGAGCTGGAAA
GAAAGAGAGCCGAGGGAGAGGTGCCGCGATATGGTCGCAAGAAAAAACAA

AAAGAAATCCGATATGATGACGAGGACGAGGATGAAGGTCCACAACCGGA
TCCTACACTTGAACAGGGTGTGCCAATACCTGTTCGATTGCAGGGCAGCT
TCCCGCCGGAATTGGCCTCCACTCCTCTCGAGGATATCGATCCCTACTAC
AGCAATGTACTGACATTCGTAGTTGTAAGCAAAGGAAAAGATATTTTTCG
CTTTTCTGCATCAAAAGCAATGTGGATGCTCGATCCATTCAATCCGATAC
GTCGTGTGGCCATTTACATTCTAGTGCATCCATTATTTTCCCTATTCATC
ATCACCACAATTCTCGTCAACTGCATCCTGATGATAATGCCGACAACGCC
CACGGTTGAGTCCACTGAGGTGATATTCACCGGAATCTACACATTTGAAT
CAGCTGTTAAAGTGATGGCACGAGGTTTCATTTTATGCCCGTTTACGTAT
CTTAGAGATGCATGGAATTGGCTGGACTTCGTAGTAATAGCTTTAGCTTA
TGTGACCATGGGTATAGATTTAGGTAATCTAGCAGCCCTGCGAACGTTTA
GGGTGCTGCGAGCGCTTAAAACCGTAGCCATTGTGCCAGGCTTGAAGACC
ATCGTCGGCGCCGTCATCGAATCGGTGAAGAATCTGCGCGATGTGATTAT
CCTGACCATGTTCTCCCTGTCGGTGTTCGCGTTGATGGGCCTACAGATCT
ATATGGGCGTGCTCACCGAGAAGTGCATCAAGAAGTTCCCGCTGGACGGT
TCCTGGGGCAATCTGACCGACGAGAACTGGGACTATCACAATCGCAATAG
CTCCAATTGGTATTCCGAGGACGAGGGCATCTCATTTCCGTTATGCGGCA
ATATATCCGGTGCGGGGCAATGCGACGACGATTACGTGTGCCTGCAGGGG
TTTGGTCCGAATCCGAATTATGGCTACACCAGCTTCGATTCGTTCGGATG
GGCTTTCCTGTCCGCCTTCCGGCTGATGACACAGGACTTCTGGGAGGATC
TGTACCAGCTGGTGTTGCGCGCCGCCGGACCATGGCACATGCTGTTCTTT
ATAGTCATCATCTTCCTAGGTTCATTCTATCTTGTGAATTTGATTTTGGC
CATTGTTGCCATGTCGTATGACGAATTGCAAAGGAAGGCCGAAGAAGAAG
AGGCTGCCGAAGAGGAGGCGATACGTGAAGCGGAAGAAGCTGCCGCCGCC
AAAGCGGCCAAGCTGGAGGAGCGGGCCAATGCGCAGGCTCAGGCAGCAGC
GGATGCGGCTGCCGCCGAAGAGGCTGCACTGCATCCGGAAATGGCCAAGA

GTCCGACGTATTCTTGCATCAGCTATGAGCTATTTGTTGGCGGCGAGAAG
GGCAACGATGACAACAACAAAGAGAAGATGTCCATTCGGAGCGTCGAGGT
GGAGTCGGAGTCGGTGAGCGTTATACAAAGACAACCAGCACCTACCACAG
CACACCAAGCTACCAAAGTTCGTAAAGTGAGCACGACATCCTTATCCTTA
CCTGGTTCACCGTTTAACATACGCAGGGGATCACGTAGTTCTCACAAGTA
CACGATACGGAACGGACGTGGCCGCTTTGGTATACCCGGTAGCGATCGTA
AGCCATTGGTATTGTCAACATATCAGGATGCCCAGCAGCACTTGCCCTAT
GCCGACGACTCGAATGCCGTCACCCCGATGTCCGAAGAGAATGGGGCCAT
CATAGTGCCCGTGTACTATGGCAATCTAGGCTCCCGACACTCATCGTATA
CCTCGCATCAGTCCCGAATATCGTATACCTCACATGGCGATCTACTCGGC
GGCATGGCCGTCATGGGCGTCAGCACAATGACCAAGGAGAGCAAATTGCG
CAACCGCAACACACGCAATCAATCAGTGGGCGCCACCAATGGCGGCACCA
CCTGTCTGGACACCAATCACAAGCTCGATCATCGCGACTACGAAATTGGC
CTGGAGTGCACGGACGAAGCTGGCAAGATTAAACATCATGACAATCCTTT
TATCGAGCCCGTCCAGACACAAACGGTGGTTGATATGAAAGATGTGATGG
TCCTGAATGACATCATCGAACAGGCCGCTGGTCGGCACAGTCGGGCAAGC
GATCGCGGTGTCTCCGTTTACTATTTCCCAACAGAGGACGATGACGAGGA
TGGGCCGACGTTCAAAGACAAGGCACTCGAAGTGATCCTCAAAGGCATCG
ATGTGTTTTGTGTGTGGGACTGTTGCTGGGTTTGGTTGAAATTTCAGGAG
TGGGTATCGCTCATCGTCTTCGATCCCTTCGTCGAGCTCTTCATCACGCT
GTGCATTGTGGTCAACACGATGTTCATGGCAATGGATCACCACGATATGA
ACAAGGAGATGGAACGCGTGCTCAAGAGTGGCAACTATTTCTTCACCGCC
ACCTTTGCCATCGAGGCCACCATGAAGCTAATGGCCATGAGCCCCAAGTA
CTATTTCCAGGAGGGCTGGAACATCTTCGACTTCATTATCGTGGCCCTAT
CGCTATTGGAACTGGGACTCGAGGGTGTCCAGGGTCTGTCCGTATTGCGT
TCCTTTCGATTGCTGCGTGTATTCAAACTGGCCAAGTCTTGGCCCACACT

TAATTTACTCATTTCGATTATGGGACGCACCATGGGCGCTTTGGGTAATC
TGACATTTGTACTTTGCATTATCATCTTCATCTTTGCGGTGATGGGAATG
CAACTGTTCGGAAAGAATTATCATGATCACAAGGACCGCTTTCCGGATGG
CGACCTGCCGCGCTGGAACTTCACCGACTTTATGCACAGCTTCATGATCG
TGTTCCGGGTGCTCTGCGGAGAATGGATCGAGTCCATGTGGGACTGCATG
TACGTGGGCGATGTCTCGTGCATTCCCTTCTTCTTGGCCACCGTTGTCAT
CGGCAATCTTGTGGTACTTAACCTTTTCTTAGCCTTGCTTTTGTCCAATT
TTGGCTCATCTAGCTTATCAGCGCCGACTGCCGATAACGATACGAATAAA
ATAGCCGAGGCCTTCAATCGAATTGGCCGATTTAAAAGTTGGGTTAAGCG
TAATATTGCTGATTGTTTCAAGTTAATACGTAACAAATTGACAAATCAAA
TAAGTGATCAACCATCAGGTGAGAGGACCAACCAGATCAGTTGGATTTGG
AGCGAAGAGCATGGTGACAACGAACTGGAGCTGGGCCACGACGAGATCCT
CGCCGACGGCCTCATCAAGAAGGGGATCAAGGAGCAGACGCAACTGGAGG
TGGCCATCGGGGATCGGATGGAATTCACGATACACGGCGACATGAAGAAC
AACAAGCCGAAGAAATCCAAATATCTAAATAACGCAACGATGATTGGCAA
CTCAATTAACCACCAAGACAATAGACTGGAACACGAGCTAAACCATAGAG
GTTTGTCCTTACAGGACGACGACACTGCCAGCATTAACTCATATGGTAGC
CATAAGAATCGACCATTCAAGGACGAGAGCCACAAGGGCAGCGCCGAGAC
GATGGAGGGCGAGGAGAAGCGCGACGCCAGCAAGGAGGATTTAGGTCTCG
ACGAGGAACTGGACGAGGAGGGCGAATGCGAGGAGGGCCCGCTCGACGGT
GATATCATTATTCATGCACACGACGAGGATATACTCGATGAATATCCAGC
TGATTGCTGCCCCGATTCGTACTATAAGAAATTTCCGATCTTAGCCGGTG
ACGATGACTCGCCGTTCTGGCAAGGATGGGGCAATTTACGACTGAAAACT
TTTCAATTAATTGAAAATAAATATTTTGAAACAGCTGTTATCACTATGAT
TTTAATGAGTAGCTTAGCTTTGGCATTAGAAGATGTACATCTGCCACAAA
GACCCATACTGCAGGATATTTTATACTATATGGACAGAATATTTACGGTT

ATATTCTTCTTGGAAATGTTAATCAAGTGGTTGGCGCTCGGCTTCAAAGT
GTACTTCACCAACGCGTGGTGTTGGCTCGATTTCGTGATTGTCATGGTAT
CGCTTATCAACTTCGTTGCTTCACTTGTTGGAGCTGGTGGTATTCAAGCC
TTCAAGACTATGCGAACGTTAAGAGCACTGAGACCACTACGTGCCATGTC
CCGTATGCAGGGCATGAGGGTCGTCGTTAATGCGCTGGTACAAGCTATAC
CGTCCATCTTCAATGTGCTATTGGTGTGTCTAATATTTTGGCTAATTTTT
GCCATAATGGGTGTACAGCTTTTTGCTGGAAAATATTTTAAGTGCGAGGA
CATGAATGGCACGAAGCTCAGCCACGAGATCATACCAAATCGCAATGCCT
GCGAGAGCGAGAACTACACGTGGGTGAATTCAGCAATGAATTTCGATCAT
GTAGGTAACGCGTATCTGTGCCTTTTCCAAGTGGCCACCTTCAAAGGCTG
GATACAAATCATGAACGATGCTATCGATTCACGAGAGGTGGACAAGCAAC
CAATTCGTGAAACGAACATCTACATGTATTTATATTTCGTATTCTTCATC
ATATTTGGATCCTTTTTCACACTCAATCTGTTCATTGGTGTTATCATTGA
TAATTTTAATGAGCAAAAGAAAAAAGCAGGTGGATCATTAGAAATGTTCA
TGACAGAAGATCAGAAAAAGTACTATAATGCTATGAAAAAGATGGGCTCT
AAAAAACCATTAAAAGCCATTCCAAGACCAAGGTGGCGACCACAAGCAAT
AGTCTTTGAAATAGTAACCGATAAGAAATTCGATATAATCATTATGTTAT
TCATTGGTCTGAACATGTTCACCATGACCCTCGATCGTTACGATGCGTCG
GACACGTATAACGCGGTCCTAGACTATCTCAATGCGATATTCGTAGTTAT
TTTCAGTTCCGAATGTCTATTAAAAATATTCGCTTTACGATATCACTATT
TTATTGAGCCATGGAATTTATTTGATGTAGTAGTTGTCATTTTATCCATC
TTAGGTCTTGTACTTAGCGATATTATCGAGAAGTACTTCGTGTCGCCGAC
CCTGCTCCGAGTGGTGCGTGTGGCGAAAGTGGGCCGTGTCCTTCGACTGG
TGAAGGGAGCCAAGGGCATTCGGACACTGCTCTTCGCGTTGGCCATGTCG
CTGCCGGCCCTGTTCAACATCTGCCTGCTGCTGTTCCTGGTCATGTTCAT
CTTTGCCATTTTCGGCATGTCGTTCTTCATGCACGTGAAGGAGAAGAGCG

GCATTAACGACGTCTACAACTTCAAGACCTTTGGCCAGAGCATGATCCTG
CTCTTTCAGATGTCGACGTCAGCCGGTTGGGATGGTGTACTGGACGCCAT
TATCAATGAGGAAGCATGCGATCCACCCGACAGCGACAAAGGCTATCCGG
GCAATTGTGGTTCAGCGACCGTTGGAATAACGTTTCTCCTCTCATACCTA
GTTATAAGCTTTTTGATAGTTATTAATATGTACATTGCTGTCATTCTCGA
GAACTATAGTCAGGCCACCGAGGACGTGCAAGAGGGTCTAACCGACGACG
ACTACGACATGTACTATGAGATCTGGCAGCAATTCGATCCGGAGGGCACC
CAGTACATACGCTATGATCAGCTGTCCGAATTCCTGGACGTACTGGAGCC
CCCGCTGCAGATCCACAAACCGAACAAGTACAAGATCATATCGATGGACA
TACCCATCTGTCGCGGTGACCTCATGTACTGCGTCGACATCCTCGACGCC
CTTACGAAAGACTTCTTTGCGCGGAAGGGCAATCCGATAGAGGAGACGGG
TGAGATTGGTGAGATAGCGGCCCGCCCGGATACGGAGGGCTACGAGCCCG
TCTCATCAACGCTGTGGCGTCAGCGTGAGGAGTACTGCGCCCGGCTAATC
CAGCACGCCTGGCGAAAGCACAAGGCGCGCGGCGAGGGAGGTGGGTCCTT
TGAGCCGGATACGGATCATGGCGATGGCGGTGATCCGGATGCCGGGGACC
CGGCGCCCGATGAAGCAACGGACGGCGATGCGCCCGCTGGTGGAGATGGT
AGTGTTAACGGTACTGCAGAAGGAGCTGCCGATGCCGATGAGAGTAATGT
AAATAGTCCGGGTGAGGATGCAGCGGCGGCGGCAGCAGCAGCAGCAGCAG
CGGCGGCGGCGGGCACGACGACGGCGGGAAGTCCCGGAGCGGGTAGCGCC
GGGCGACAGACCGCCGTTCTCGTGGAGAGCGACGGGTTCGTGACGAAGAA
CGGCCACAAGGTGGTCATCCACTCGCGATCGCCGAGCATCACGTCGCGCA
CGGCGGATGTCTGAGCCAGGCCTCGCCCCCCCCTCCAAGATGCACGCGAG
TATTAGCTCTAGA [SEQ.ID.NO.:7].

EXAMPLE 2

In Vitro Synthesis of *para* and *tipE* Synthetic mRNA for *In Vitro* or *In Vivo* Translation

The protocol for the production of *para* and *tipE* synthetic mRNA is identical. Synthetic mRNA is produced in sufficient quantity *in vitro* by cloning double stranded DNA encoding *para* and *tipE* mRNA into a plasmid vector containing a bacteriophage promoter, linearizing the plasmid vector containing the cloned *para*-encoding DNA, and transcribing the cloned DNA *in vitro* using a DNA-dependent RNA polymerase from a bacteriophage that specifically recognizes the bacteriophage promoter on the plasmid vector.

Various plasmid vectors are available containing a bacteriophage promoter recognized by a bacteriophage DNA-dependent RNA polymerase, including but not limited to plasmids pSP64, pSP65, pSP70, pSP71, pSP72, pSP73, pGEM-3Z, pGEM-4Z, pGEM-3Zf, pGEM-5Zf, pGEM-7Zf, pGEM-9Zf, and pGEM-11Zf, the entire series of plasmids is commercially available from Promega.

It may be advantageous to synthesize mRNA containing a 5' terminal cap structure and a 3' poly A tail to improve mRNA stability. A cap structure, or 7-methylguanosine, may be incorporated at the 5' terminus of the mRNA by simply adding 7-methylguanosine to the reaction mixture with the DNA template. The DNA-dependent RNA polymerase incorporates the cap structure at the 5' terminus as it synthesizes the mRNA. The poly-A tail is found naturally occurring in many cDNAs but can be added to the 3' terminus of the mRNA by simply inserting a poly A tail-encoding DNA sequence at the 3' end of the DNA template.

The 6513 bp double stranded *para* encoding DNA was subcloned into the bacteriophage containing vector pGH19 as described in Figure 2. The pGH19 vector was derived from of the pGEMHE vector (Liman *et al.*, 1992, Subunit stiochiometry of a mammalian K+ Channel determined by construction of multimeric cDNAs. *Neuron* 9:861-871) by inserting NotI and XhoI restriction enzyme sites between

the unique PstI and NheI sites of pGEMHE (Evan Goulding and Steve Siegelbaum, Columbia University). The plasmid vector containing the cloned *para*-encoding DNA was linearized with the restriction enzyme NotI and *in vitro* synthesized *para* mRNA containing a 5' terminal cap structure was synthesized using either the mMessage mMachine (Ambion) or mCAP (Stratagene) kits per manufacturer's instructions.

The isolated and purified *para* and *tipE* mRNA is translated using either a cell-free system, including but not limited to rabbit reticulocyte lysate and wheat germ extracts (both commercially available from Promega and New England Nuclear) or in a cell based system, including but not limited to microinjection into Xenopus oocytes, with microinjection into Xenopus oocytes being preferred.

Xenopus oocytes were microinjected with a sufficient amount of synthetic *para* and *tipE* mRNA to produce *para* and *tipE* protein. The synthetic *para* and *tipE* mRNAs were injected into Xenopus oocytes by standard procedures and were analyzed for *para* and *tipE* expression as described below.

EXAMPLE 3

Characterization Of *para* voltage-activated sodium channels in Xenopus oocytes

Xenopus laevis oocytes were prepared and injected using standard methods previously described and known in the art [Arena, J.P., Liu, K.K., Paress, P.S. & Cully, D.F. *Mol. Pharmacol.* 40, 368-374 (1991); Arena, J.P., Liu, K.K., Paress, P.S., Schaeffer, J.M. & Cully, D.F. *Mol. Brain Res.* 15, 339-348 (1992)]. Adult female Xenopus laevis were anesthetized with 0.17% tricaine methanesulfonate and the ovaries were surgically removed and placed in a dish consisting of (mM): NaCl 82.5, KCl 2, $MgCl_2$ 1, $CaCl_2$ 1.8, HEPES 5 adjusted to pH 7.5 with NaOH (OR-2). Ovarian lobes were broken open, rinsed several times, and gently shaken in OR-2 containing 0.2% collagenase (Sigma, Type 1A) for 2-5 hours. When approximately 50% of the follicular layers were removed, Stage V and VI oocytes were selected and placed in media consisting of (mM): NaCl 86, KCl 2, $MgCl_2$ 1,

$CaCl_2$ 1.8, HEPES 5, Na pyruvate 2.5, theophylline 0.5, gentamicin 0.1 adjusted to pH 7.5 with NaOH (ND-96) for 24-48 hours before injection. Oocytes were injected with 50 nl of *para* RNA (50-250 ng) and/or *tipE* RNA (50-250 ng). Control oocytes were injected with 50 nl of water. Oocytes were incubated for 2-10 days in ND-96 before recording. Incubations and collagenase digestion were carried out at 18°C.

Recordings were made at room temperature 2-10 days after injection in standard frog saline consisting of (mM): NaCl 115, KCl 2, $MgCl_2$ 1, $CaCl_2$ 1.8, HEPES 10 adjusted to pH 7.5 with NaOH. Oocytes were voltage-clamped using a standard two microelectrode amplifier (Dagan 8500 or TEV-200, Minneapolis, MN). Pipettes were filled with 3 M KCl and had resistance's between 0.5-3.0 MΩ. The Plexiglas recording chamber (volume 200 μl) was connected to ground with a Ag/AgCl electrode. Data were acquired and analyzed using the PCLAMP software package with a TL-1 interface (Axon Instruments, Foster City, CA). The amplitude of peak voltage-activated sodium currents were determined after subtraction of linear leak currents, or as the tetrodotoxin-sensitive determined after subtraction of the current in the presence of 30 nM tetrodotoxin. Data were filtered at 2-5 kHz and sampled at 10-33 kHz.

Oocytes injected with *in vitro* RNA for *para* and *tipE* expressed voltage-activated sodium currents (Fig. 3). Currents were elicited with 20 sec voltage steps from a holding potential of -100 mV (voltage protocol depicted in Fig. 3a). Oocytes simultaneously expressing *para* and *tipE* proteins exhibited the rapidly activating and inactivating inward currents (Fig. 3b). The threshold for current activation was approximately -33 ± 3 mV (n=6), and peak currents were observed at -3 ± 2 mV (n=6). The voltage-activated currents were completely inhibited with 10 nM tetrodotoxin (Fig 3 Panels B and C, n=10). The voltage-dependence of inactivation was also examined (Fig. 4). Test pulses to 0 mV were preceded by 50 msec prepulses to the potentials indicated on the abscissa (Fig. 4). Normalized peak current was plotted as a function of the prepulses potential. The smooth curve

is a fit of the data to the function $I=\{1+\exp[(V_m-V_{1/2})/k]\}^{-1}$ where I is the normalized current, V_m is the prepulse potential, $V_{1/2}$ is the point of half-maximal inactivation, and k is the slope factor. $V_{1/2}$ was -42 ± 1 mV with a slope factor of 5.2 ± 0.5 (n=4).

Several lines of evidence demonstrate that the current expressed after coinjection of *para* and *tipE in vitro* RNA represents Drosophila voltage-activated sodium currents. First, the current is blocked with tetrodotoxin, a potent selective inhibitor of vertebrate and invertebrate voltage-activated sodium channels [Catterall, W.A. Ann. *Rev. Pharmacol. Toxicol.* 20, 15-43 (1980)]. Similar to the *para* sodium currents expressed in oocytes, the sodium currents recorded from Drosophila embryonic neurons are completely inhibited with 10 nM tetrodotoxin [O'Dowd, D.K. and Aldrich, R.W. *J. Neurosci.* 8, 3633-3643 (1988); Saito, M. and Wu, C.F. *J. Neurosci.* 11, 2135-2150 (1991)]. Secondly, very rapid activation and inactivation of the current, the threshold for activation, and the voltage dependence of peak current agree with data previously reported from Drosophila neurons in culture [O'Dowd, D.K. and Aldrich, R.W. *J. Neurosci.* 8, 3633-3643 (1988); Byerly, L. and Leung, H.T. *J. Neurosci.* 8, 4379-4393 (1988); Saito, M. and Wu, C.F. *J. Neurosci.* 11, 2135-2150 (1991)]. Finally, the $V_{1/2}$ and slope of the steady-state inactivation curve was very close to that reported for Drosophila embryonic neurons [O'Dowd, D.K. and Aldrich, R.W. *J. Neurosci.* 8, 3633-3643 (1988)].

Injection of the individual subunits, *para* or *tipE*, failed to express functional homomeric channels. Injection of oocytes with 200-300 ng of an individual subunit RNA resulted in no voltage-activated sodium current for up to 8 days after injection. In contrast, after coinjection of 150 ng of both subunits 50 % of the oocytes express voltage-activated sodium currents after 3 days, and 90 % on day 5.

EXAMPLE 4

Cloning of the *para* and *tipE* cDNA into *E. coli* Expression Vectors

The protocol for the expression of *para* and *tipE* in *E. coli* is identical. Recombinant *para* is produced in *E. coli* following the transfer of the *para* expression cassette into *E. coli* expression vectors, including but not limited to, the pET series (Novagen). The pET vectors place *para* expression under control of the tightly regulated bacteriophage T7 promoter. Following transfer of this construct into an *E. coli* host which contains a chromosomal copy of the T7 RNA polymerase gene driven by the inducible lac promoter, expression of *para* is induced when an approriate lac substrate (IPTG) is added to the culture. The levels of expressed *para* are determined by the assays described above.

The cDNA encoding the entire open reading frame for *para* or *tipE* is inserted into the NdeI site of pET [16]11a. Constructs in the positive orientation are identified by sequence analysis and used to transform the expression host strain BL21. Transformants are then used to inoculate cultures for the production of *para* and *tipE* protein. Cultures may be grown in M9 or ZB media, whose formulation is known to those skilled in the art. After growth to an approximate OD600= 1.5, expression of *para* or *tipE* is induced with about 1 mM IPTG for about 3 hours at 37°C.

EXAMPLE 5

Cloning of *para* and *tipE* cDNA into Mammalian Expression Vectors

Para and *tipE* cDNA expression cassettes are ligated at appropriate restriction endonuclease sites to vectors containing strong, universal mammalian promoters, including but not limited to: pcDNA3 (Invitrogen), pBC12BI [Cullen, B.R. *Methods in Enzymol.* 152: 684-704 1988], and pEE12 (CellTech EP O 338,841), or strong inducible mammalian promoters, including but not limited to, pMAMneo (Clontech).

Cassettes containing the *para* and *tipE* cDNA in the positive orientation with respect to the promoter are ligated into appropriate restriction sites 3' of the promoter and identified by restriction site mapping and/or sequencing. These cDNA expression vectors are introduced into various host cells including, but not limited to: COS-7 (ATCC# CRL1651), CV-1 [Sackevitz *et al., Science* 238: 1575 (1987)], 293, L cells (ATCC# CRL6362)] by standard methods including but not limited to electroporation, or chemical procedures (cationic liposomes, DEAE dextran, calcium phosphate). Transfected cells and cell culture extracts can be harvested and analyzed for *para* and *tipE* expression as described below.

All of the vectors used for mammalian transient expression can be used to establish stable cell lines expressing *para* and *tipE*. Unaltered *para* and *tipE* cDNA constructs cloned into expression vectors will be expected to program host cells to make intracellular *para* and *tipE* protein. The transfection host cells include, but are not limited to, CV-1 [Sackevitz *et al., Science* 238: 1575 (1987)], tk-L [Wigler, *et al. Cell* 11: 223 (1977)], NS/0, and dHFr-CHO [Kaufman and Sharp, *J. Mol. Biol.* 159: 601, (1982)].

Co-transfection of any vector containing *para* and *tipE* cDNA with a drug selection plasmid including, but not limited to G418, aminoglycoside phosphotransferase, pLNCX [Miller, A.D. and Rosman G. J. *Biotech News* 7: 980-990 (1989)]; hygromycin, hygromycin-B phosphotransferase, pLG90 [Gritz. L. and Davies, J., *GENE* 25: 179 (1983)] ; APRT, xanthine-guanine phosphoribosyl-transferase, pMAM (Clontech) [Murray, *et al., Gene* 31: 233 (1984)] will allow for the selection of stably transfected clones. Levels of *para* and *tipE* are quantitated by the assays described above.

Para and *tipE* cDNA constructs are ligated into vectors containing amplifiable drug-resistance markers for the production of mammalian cell clones synthesizing the highest possible levels of *para* and *tipE*. Following introduction of these constructs into cells, clones containing the plasmid are selected with the appropriate agent, and isolation of an over-expressing clone with a high copy number

of the plasmid is accomplished by selection in increasing doses of the agent.

Cells are transfected with *para*, *tipE* or both *para* and *tipE*. Stable cell clones are selected by growth in the presence of the appropriate selectable marker. Single resistant clones are isolated and shown to contain the intact *para* or *tipE* gene or both *para* and *tipE* genes. Clones containing the *para* and *tipE* cDNAs are analyzed for expression using immunological techniques, such as immune-precipitation, Western blot, and immunofluorescence using antibodies specific to the *para* and *tipE* proteins. Antibody is obtained from rabbits inoculated with peptides that are synthesized from the amino acid sequence predicted from the *para* and *tipE* sequences. Expression is also analyzed using patch clamp electrophysiological techniques and ^{3}H-saxitoxin binding assays.

Cells that are expressing *para* and *tipE*, stably or transiently, are used to test for expression of voltage-activated sodium channels and for ligand binding activity. These cells are used to identify and examine other compounds for their ability to modulate, inhibit or activate the *para* voltage-activated sodium channel as described herein.

Cloning of *para* and *tipE* cDNA into Drosophila Expression Vectors

Para and *tipE* cDNA expression cassettes are ligated at appropriate restriction endonuclease sites to vectors containing constituted or inducible Drosophila promoters, including but not limited to: pRmHa-1 (Bunch *et al.*, 1988, Characterization and use of the Drosophila metallothionein promoter in cultured Drosophila melanogaster cells. *Nucleic Acids Research* 16:1043-1060) and pCaSpeR-act (Thummel *et al.*, 1988, Vectors for Drosophila P-element-mediated transformation and tissue culture transfection *Gene* 74:445-456).

Cassettes containing the *para* and *tipE* cDNA in the positive orientation with respect to the promoter are ligated into appropriate restriction sites 3' of the promoter and identified by restriction site mapping and/or sequencing. These cDNA expression vectors are

introduced into various host cells including, but not limited to: Schneider-2 and Kc cells by standard methods including but not limited to electroporation, or chemical procedures (cationic liposomes, DEAE dextran, calcium phosphate). Transfected cells and cell culture extracts can be harvested and analyzed for *para* and *tipE* expression as described herein.

All of the vectors used for <u>Drosophila</u> transient expression can be used to establish stable cell lines expressing *para* and *tipE*. Unaltered *para* and *tipE* cDNA constructs cloned into expression vectors will be expected to program host cells to make intracellular *para* and *tipE* protein.

Co-transfection of any vector containing *para* and *tipE* cDNA with a drug selection plasmid including, but not limited to G418, aminoglycoside phosphotransferase, [Miller, A.D. and Rosman G. J. *Biotech News* 7: 980-990 (1989)]; and hygromycin, hygromycin-B phosphotransferase, [Gritz. L. and Davies, J., *GENE* 25: 179 (1983)] will allow for the selection of stably transfected clones. Levels of *para* and *tipE* are quantitated by the assays described above.

para and *tipE* cDNA constructs are ligated into vectors containing amplifiable drug-resistance markers for the production of <u>Drosophila</u> cell clones synthesizing the highest possible levels of *para* and *tipE*. Following introduction of these constructs into cells, clones containing the plasmid are selected with the appropriate agent, and isolation of an over-expressing clone with a high copy number of the plasmid is accomplished by selection in increasing doses of the agent.

Cells are transfected with *para*, *tipE* or both *para* and *tipE*. Stable cell clones are selected by growth in the presence of the appropriate selectable marker. Single resistant clones are isolated and shown to contain the intact *para* or *tipE* gene or both *para* and *tipE* genes. Clones containing the *para* and *tipE* cDNAs are analyzed for expression using immunological techniques, such as immune-precipitation, Western blot, and immunofluorescence using antibodies specific to the *para* and *tipE* proteins. Antibody is obtained from rabbits inoculated with peptides that are synthesized from the amino

acid sequence predicted from the *para* and *tipE* sequences. Expression is also analyzed using patch clamp electrophysiological techniques and ^{3}H-saxitoxin binding assays.

Cells that are expressing *para* and *tipE*, stably or transiently, are used to test for expression of voltage-activated sodium channels and for ligand binding activity. These cells are used to identify and examine other compounds for their ability to modulate, inhibit or activate the *para* voltage-activated sodium channel as described herein.

EXAMPLE 6

Cloning of *para* and *tipE* cDNA into a Baculovirus Expression Vector for Expression in Insect Cells

Baculovirus vectors, which are derived from the genome of the AcNPV virus, are designed to provide high level expression of cDNA in the Sf9 line of insect cells (ATCC CRL# 1711). Recombinant baculoviruses expressing *para* and/or *tipE* cDNA are produced by the following standard methods (InVitrogen Maxbac Manual): the *para* and *tipE* cDNA constructs are ligated downstream of the polyhedrin promoter in a variety of baculovirus transfer vectors, including the pAC360 and the pBlueBac vector (InVitrogen). Recombinant baculoviruses are generated by homologous recombination following co-transfection of the baculovirus transfer vector and linearized AcNPV genomic DNA [Kitts, P.A., *Nuc. Acid. Res.* 18: 5667 (1990)] into Sf9 cells. Recombinant pAC360 viruses are identified by the absence of inclusion bodies in infected cells (Summers, M. D. and Smith, G. E., Texas Agriculture Exp. Station Bulletin No. 1555) and recombinant pBlueBac viruses are identified on the basis of β-galactosidase expression (Vialard, *et al.* 1990, *J. Virol.*, 64, pp 37-50). Following plaque purification and infection of sf9 cells with *para* and/or *tipE* recombinant baculovirus, *para* and *tipE* expression is measured by the assays described herein.

The cDNA encoding the entire open reading frame for *para* or *tipE* is inserted into the BamHI site of pBlueBacII. Constructs in the

positive orientation with respect to the polyhedrin promoter are identified by sequence analysis and used to transfect Sf9 cells in the presence of linear AcNPV mild type DNA.

Authentic, active *para* and *tipE* is found associated with the membranes of infected cells. Membrane preparations are prepared from infected cells by standard procedures.

EXAMPLE 7

Cloning of *para* and *tipE* cDNA into a yeast expression vector

Recombinant *para* and *tipE* is produced in the yeast *S. cerevisiae* following the insertion of the optimal *para* and *tipE* cDNA construct into expression vectors designed to direct the intracellular expression of heterologous proteins. For intracellular expression, vectors such as EmBLyex4 or the like are ligated to the *para* or *tipE* cistron [Rinas, U. *et al., Biotechnology* 8: 543-545 (1990); Horowitz B. *et al., J. Biol. Chem.* 265: 4189-4192 (1989)]. The levels of expressed *para* and *tipE* are determined by the assays described herein.

EXAMPLE 8

Purification of Recombinant *para* and *tipE*

Recombinantly produced *para* and *tipE* may be purified by antibody affinity chromatography.

para or *tipE* antibody affinity columns are made by adding the anti-*para* or anti-*tipE* antibodies to Affigel-10 (Biorad), a gel support which is pre-activated with N-hydroxysuccinimide esters such that the antibodies form covalent linkages with the agarose gel bead support. The antibodies are then coupled to the gel via amide bonds with the spacer arm. The remaining activated esters are then quenched with 1M ethanolamine HCl (pH 8). The column is washed with water followed by 0.23 M glycine HCl (pH 2.6) to remove any non-conjugated antibody or extraneous protein. The column is then equilibrated in phosphate buffered saline (pH 7.3) together with appropriate membrane solubilizing agents such as detergents and the

cell culture supernatants or cell extracts containing solubilized *para* or *tipE* are slowly passed through the column. The column is then washed with phosphate- buffered saline together with detergents until the optical density (A280) falls to background, then the protein is eluted with 0.23 M glycine-HCl (pH 2.6) together with detergents. The purified *para* or *tipE* protein is then dialyzed against phosphate buffered saline together with detergents.

EXAMPLE 9

Assay for the identification of *para* voltage-activated sodium channel modulators

Modulators of insect sodium channels can be identified by screening for modulators of the *para* voltage-activated sodium channel. Modulators of insect sodium channel activity can be identified by a variety of approaches, including but not limited to, radioisotopic flux assays, ligand binding assays, and cell viability assays.

Measuring Na channel activity in cell populations by monitoring radioisotopic flux is a well established technique (Catterall, W.A. *Journal of Biological Chemistry* 252, 8669-8676 (1977); Tamkun, M.M. & Caterall, W.A. *Molecular Pharmacology* 19, 78-86 (1981)). Using transfected cell lines (see above) expressing the *para* voltage-activated sodium channel, modulators of the Drosophila voltage-activated sodium channel are isolated in a [22Na] flux assay in a 96-well format. To identify sodium channel agonists, *para* transfected cells are aliquoted into each well of a 96-well microtiter dish and [22Na] is added to the culture media, test compounds are added to each well and agonists are identified by an increase in [22Na] uptake as compared to untreated cells. Specificity is determined by blocking [22Na] uptake with tetrodotoxin. Likewise, sodium channel antagonist can be identified by screening for compounds that block [22Na] uptake following activation of the *para* voltage-activated sodium channel.

Sodium channel modulators can also be identified by measuring the toxicity of Na channel activators on *para* expressing cells.

Sodium channel activators are toxic because prolonged activation of sodium channels causes osmotic lysis of the cells. Sodium channel blockers are detected by their ability to protect from the toxicity of Na channel activators (Manger, R.L., Leja, L.S., Lee, S.Y., Hungerford, J.M. & Wekell, M.M. *Analytical Biochemistry* 214, 190-194 (1993)). The assay is performed in 96 well plates and toxicity is measured by employing a plate reader with a membrane-impermeant reporter, such as ethidium bromide homodimer (the methodology is described in a product application note from Molecular Probes for the Live/Dead Eukolight Cytotoxicity kit). The specificity of sodium channel activators is determined by blocking toxicity with tetrodotoxin, a highly potent and selective sodium channel blocker.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Warmke, Jeffrey W.
Van Der Ploeg, Leonardus

(ii) TITLE OF INVENTION: PROCESS FOR FUNCTIONAL EXPRESSION OF THE PARA SODIUM CHANNEL

(iii) NUMBER OF SEQUENCES: 7

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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
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(B) REGISTRATION NUMBER: 32,633
(C) REFERENCE/DOCKET NUMBER: 19338

(ix) TELECOMMUNICATION INFORMATION:
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(B) TELEFAX: (908) 594-4720

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

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(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

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(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

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(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6513 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

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ACTCGCGATC GCCCGAGCATC ACGTCGCGCA CGGCGGATGT CTGAGCCAGG CCTCGCCCCC 6480
CCCTCCAAGA TGCACGCGAG TATTAGCTCT AGA 6513

WHAT IS CLAIMED IS:

1 A monospecific antibody immunologically reactive with a voltage-activated cation channel.

2. The antibody of Claim 1, wherein the antibody blocks activity of the voltage-activated cation channel.

3. A method of identifying compounds that modulate voltage-activated cation channel activity, comprising combining a modulator of voltage-activated cation channel activity with a cell expressing a recombinant voltage-activated cation channel protein, and measuring an effect of the modulator on the channel.

4. The method of Claim 3, wherein the effect of the modulator on the channel is inhibiting or enhancing binding of voltage-activated cation channel ligands.

5. The method of Claim 3, wherein the effect of the modulator on the channel is inhibition or enhancement of cation flux mediated by voltage-activated cation channels.

6. The method of Claim 5, wherein the cation flux is membrane sodium flux.

7. A compound active in the method of Claim 3, wherein said compound is a modulator of a voltage-activated cation channel.

8. A compound active in the method of Claim 3, wherein said compound is an agonist or antagonist of a voltage-activated cation channel.

9. A compound active in the method of Claim 3, wherein said compound is a modulator of expression of a voltage-activated cation channel.

10. A pharmaceutical composition comprising a compound active in the method of Claim 3, wherein said compound is a modulator of voltage-activated cation channel activity.

11. A method of treating a patient in need of such treatment for a condition which is mediated by a voltage-activated cation channel, comprising administration of a voltage-activated cation channel modulating compound active in the method of Claim 3.

12. A method of treating a patient in need of such treatment for a condition which is mediated by a voltage-activated cation channel and is characterized by infection or infestation with an invertebrate organism, comprising administration of a voltage-activated cation channel modulating compound active in the method of Claim 3.

13. The method of Claim 12 wherein said patient is a domestic animal or livestock.

14. An agricultural composition comprising a compound active in the method of Claim 3, wherein said compound is a modulator of voltage-activated cation channel activity.

15. A method of treating an insect infestation of crops or forestry stock, comprising administration of an agricultural composition of claim 14.

16. The method of Claim 15 wherein said insects are arthropods.

17. A DNA molecule characterized by the nucleotide sequence as set forth in SEQ ID NO:7.

18 An expression vector containing the DNA molecule of Claim 17.

19. A recombinant host cell containing the expression vector of Claim 18.

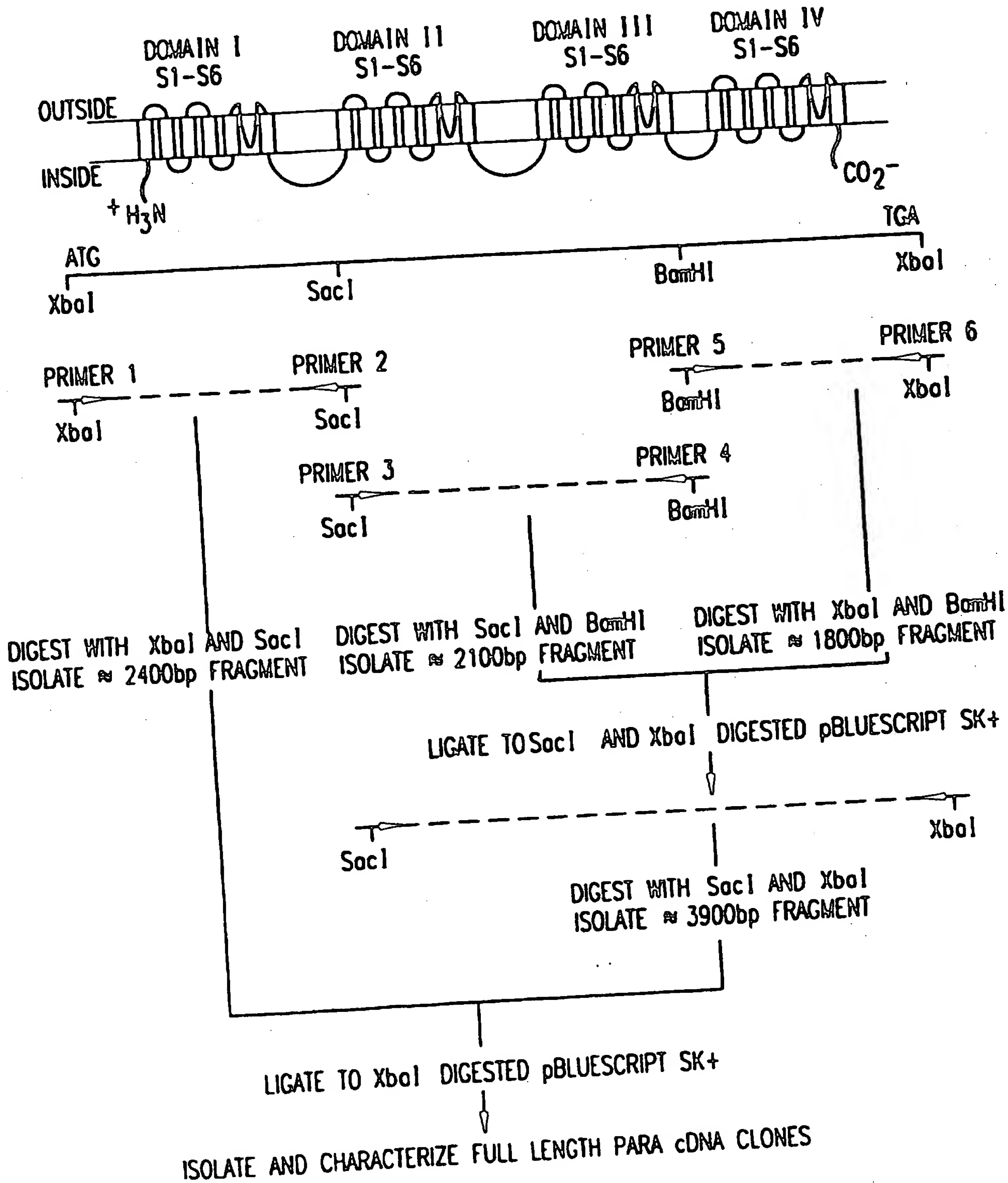

DOMAIN I
S1-S6
DOMAIN II
S1-S6
DOMAIN III
S1-S6
DOMAIN IV
S1-S6
OUTSIDE
INSIDE
^+H_3N
CO_2^-
ATG
TGA
XbaI
SacI
BamHI
XbaI
PRIMER 1
XbaI
PRIMER 2
SacI
PRIMER 5
BamHI
PRIMER 6
XbaI
PRIMER 3
SacI
PRIMER 4
BamHI
DIGEST WITH XbaI AND SacI
ISOLATE ≈ 2400bp FRAGMENT
DIGEST WITH SacI AND BamHI
ISOLATE ≈ 2100bp FRAGMENT
DIGEST WITH XbaI AND BamHI
ISOLATE ≈ 1800bp FRAGMENT
LIGATE TO SacI AND XbaI DIGESTED pBLUESCRIPT SK+
SacI
XbaI
DIGEST WITH SacI AND XbaI
ISOLATE ≈ 3900bp FRAGMENT
LIGATE TO XbaI DIGESTED pBLUESCRIPT SK+
ISOLATE AND CHARACTERIZE FULL LENGTH PARA cDNA CLONES

FIG. 1

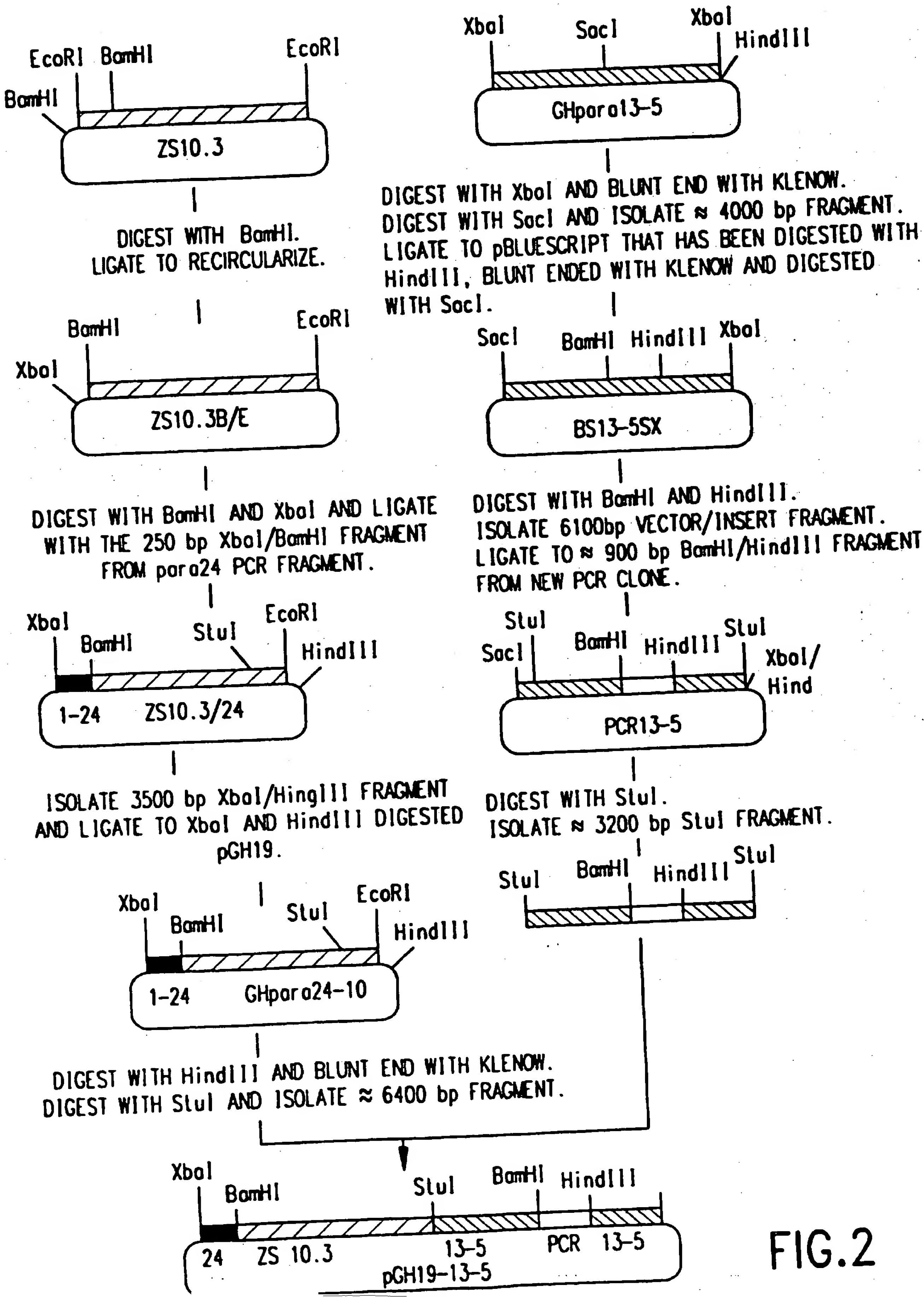

EcoRI BamHI EcoRI
BamHI
ZS10.3
DIGEST WITH BamHI.
LIGATE TO RECIRCULARIZE.
BamHI EcoRI
XbaI
ZS10.3B/E
DIGEST WITH BamHI AND XbaI AND LIGATE
WITH THE 250 bp XbaI/BamHI FRAGMENT
FROM para24 PCR FRAGMENT.
XbaI BamHI StuI EcoRI HindIII
1-24 ZS10.3/24
ISOLATE 3500 bp XbaI/HinglII FRAGMENT
AND LIGATE TO XbaI AND HindIII DIGESTED
pGH19.
XbaI BamHI StuI EcoRI HindIII
1-24 GHpara24-10
DIGEST WITH HindIII AND BLUNT END WITH KLENOW.
DIGEST WITH StuI AND ISOLATE ≈ 6400 bp FRAGMENT.
XbaI SacI XbaI HindIII
GHpara13-5
DIGEST WITH XbaI AND BLUNT END WITH KLENOW.
DIGEST WITH SacI AND ISOLATE ≈ 4000 bp FRAGMENT.
LIGATE TO pBLUESCRIPT THAT HAS BEEN DIGESTED WITH
HindIII, BLUNT ENDED WITH KLENOW AND DIGESTED
WITH SacI.
SacI BamHI HindIII XbaI
BS13-5SX
DIGEST WITH BamHI AND HindIII.
ISOLATE 6100bp VECTOR/INSERT FRAGMENT.
LIGATE TO ≈ 900 bp BamHI/HindIII FRAGMENT
FROM NEW PCR CLONE.
StuI SacI BamHI HindIII StuI XbaI/ Hind
PCR13-5
DIGEST WITH StuI.
ISOLATE ≈ 3200 bp StuI FRAGMENT.
StuI BamHI HindIII StuI
XbaI BamHI StuI BamHI HindIII
24 ZS 10.3 13-5 PCR 13-5
pGH19-13-5

FIG.2

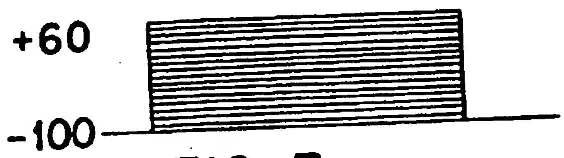


FIG. 3a

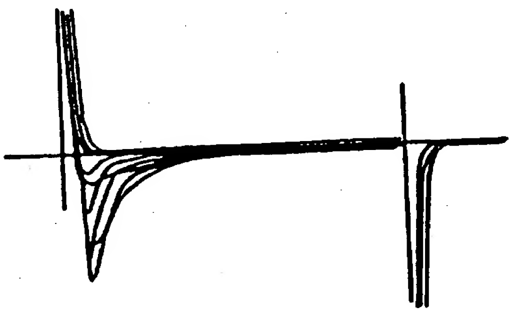


FIG. 3b

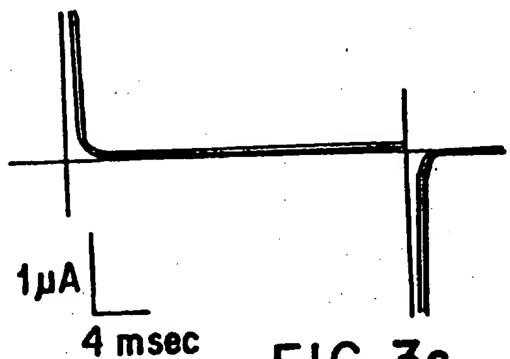


FIG. 3c

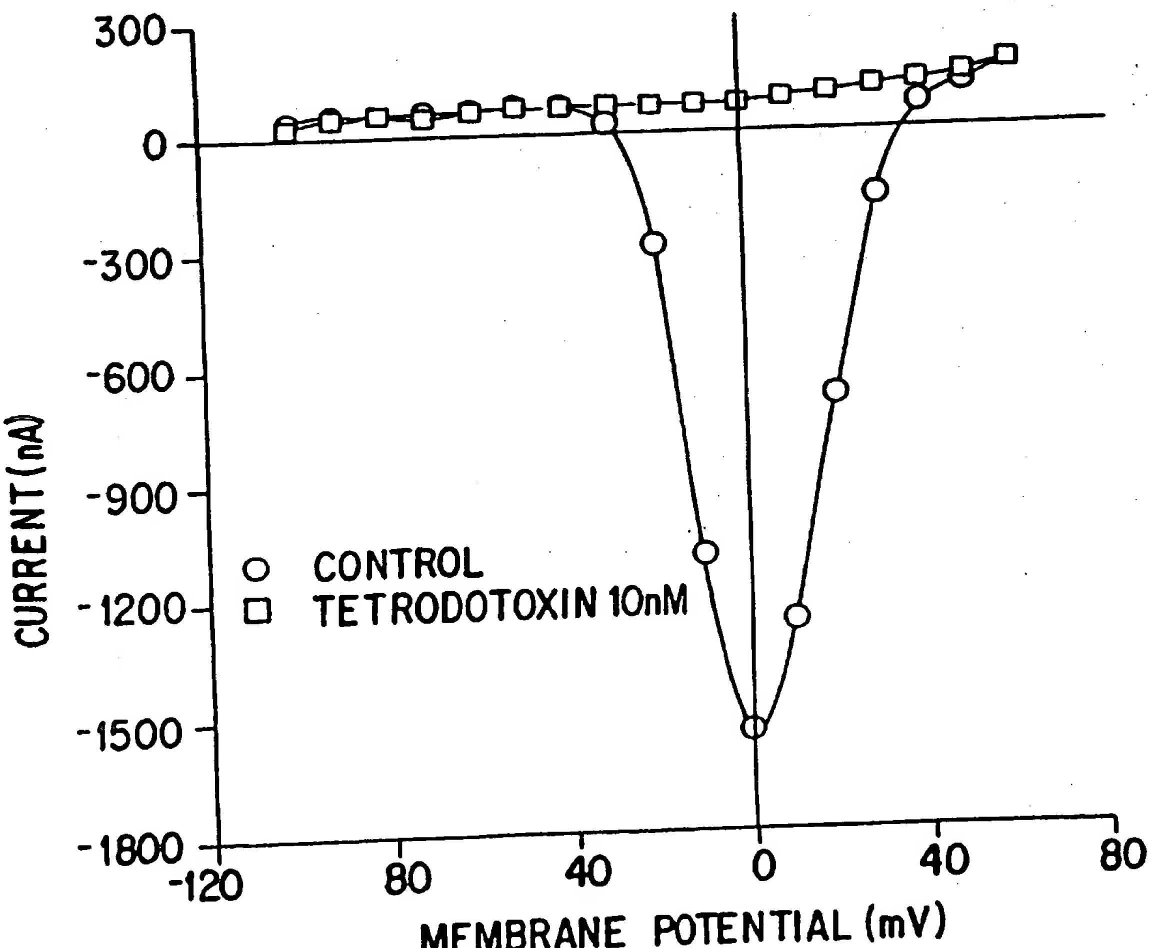
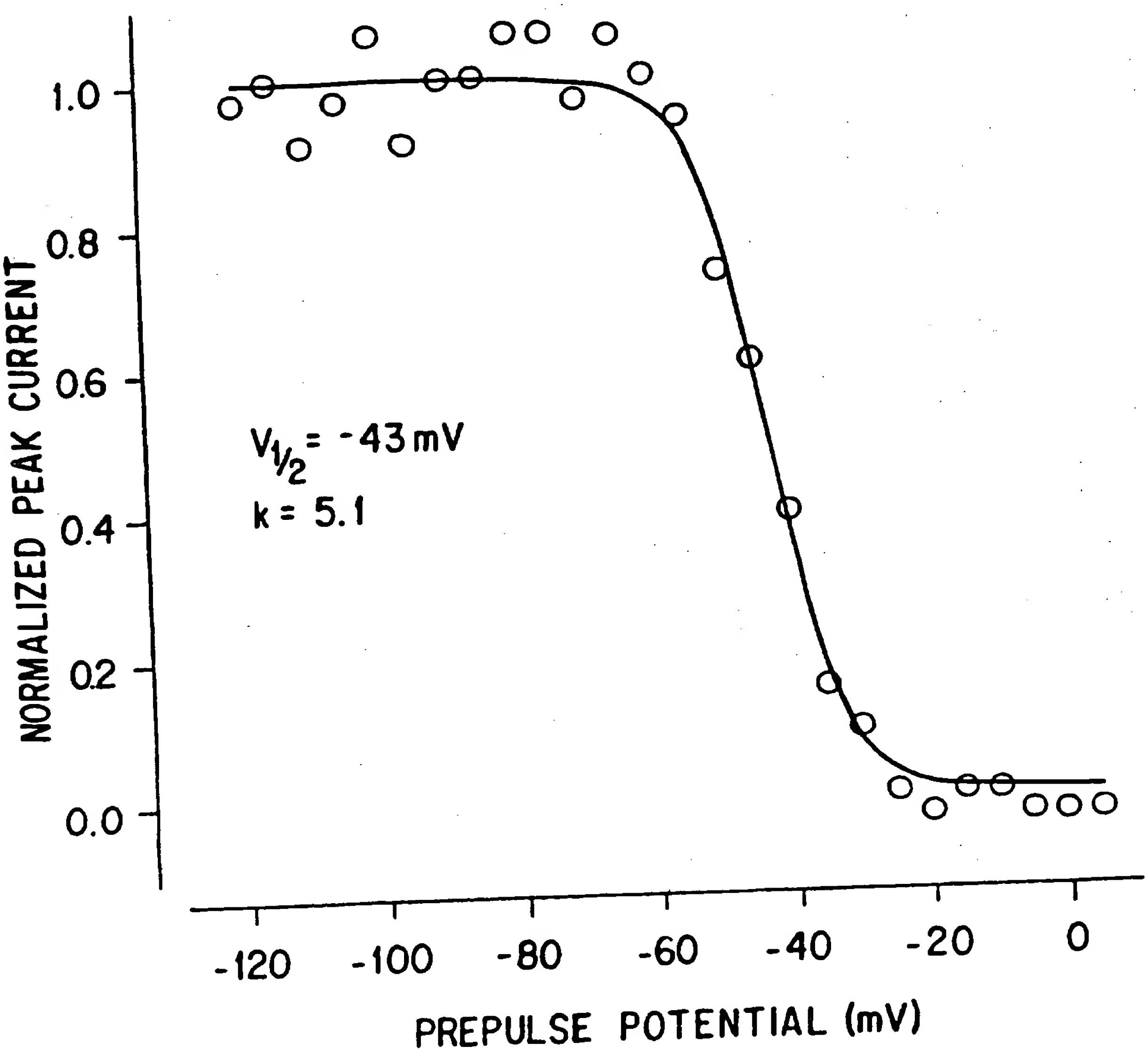


FIG. 3d



1.0
0.8
0.6
0.4
0.2
0.0
NORMALIZED PEAK CURRENT
$V_{1/2}$ = -43 mV
k = 5.1
-120
-100
-80
-60
-40
-20
0
PREPULSE POTENTIAL (mV)

FIG. 4

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/14262

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/350, 387.1, 387.9, 388.2; 435/7.21, 7.2, 320.1, 240.1, 252.3, 254.11; 424/ 139.1, 152.1; 514/2, 12; 536/23.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim N .
X	Proceedings of the National Academy of Sciences, USA, Volume 81, issued October 1984, J.M. Casadei et al., "Monoclonal antibodies against the voltage sensitive Na+ channel from mammalian skeletal muscle", pages 6227-6231, entire document.	1-2, 10
X	US, A, 3,898,339 (ADAMS ET AL.) 05 August 1975, entire document	7-8, 10
X	US, A, 4,536,591 (PLUMMER) 20 August 1985, entire document.	7, 8, 14-16

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search 05 FEBRUARY 1996	Date of mailing of the international search report 05 MAR 1996
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer GABRIELE E. BUGAISKY Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Journal of Clinical Investigation, Volume 83, Number 5, issued May 1989, G.F. Tomaselli et al., "Sodium channels from human brain expressed in Xenopus oocytes. Basic electrophysiologic characteristics and their modification by diphenylhydantoin.", pages 1724-1732, entire document.	3-8, 10
X	Pfluegers Archiv European Journal of Physiology, Volume 426, Numbers 3-4, issued 1994, W. Schreibmayer et al., "Mechanism of modulation of single sodium channels from skeletal muscle by the beta-1 subunit from rat brain", pages 360-362, entire document.	3-8
X	Nature, Volume 322, issued 28 August 1986, M. Noda et al., "Expression of functional sodium channels from cloned cDNA", pages 826-828, especially figure 3.	3-8
A	Journal of Neuroscience, Volume 14, Number 5, issued May 1994, J.R. Thackeray et al., "Developmentally regulated alternative splicing generates a complex array of Drosophila para sodium channel isoforms", pages 2569-2578, entire document.	17-19
X	Science, Volume 231, issued 07 March 1988, N. Dascal et al., "Expression and modulation of voltage-gated calcium channels after RNA injection in Xenopus oocytes", pages 1147-1150, entire document.	3-8, 10
X	European Journal of Biochemistry, Volume 216, Number 3, issued 15 September 1993, S. Reinhardt-Maelicke et al., "Application of an ectopic expression system for the selection of protein-isoform-specific antibodies. The monoclonal antibody K1C3 is specific for the RCK1 potassium channel.", pages 871-877, entire document.	1-8, 10

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/14262

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☒ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
1-8, 10, 14-19

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest
☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet(1))(July 1992)☆

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/14262

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

A61K 38/17, 39/395; G01N 33/566; C12N 1/15, 1/21, 5/10, 15/2, 15/63; C07K 14/435, 16/28

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

530/350, 387.1, 388.2; 435/7.21, 320.1, 240.1, 252.3, 254.11; 424/ 139.1, 152.1; 514/12; 536/23.5

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, DIALOG (files 155, 5, 357 -MEDLINE, BIOSIS, DERWENT BIOTECHNOLOGY ABSTRACTS)
search terms: cation, sodium, potassium, calcium, glutamate, channel?, voltage, gat?, activat?, monoclonal, monospecific, gene? ?, RNA, plasmid, screen?, select?, agonist, antagonist, inhibit?, insect?, xenopus, oocyte, express?, membrane, ligand, agricultur? field, infest?, modulat?

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claim(s)1-8, 10, and 14, drawn to channel antagonists and agonists, specifically monoclonal antibodies and a method for their identification.

Group II, claim(s) 9, drawn to a compound which modulates expression of ion channel genes.

Group III, claim(s) 11-13, drawn to a method of treating patients with compounds affecting ion channel activity.

Group IV, claim(s) 15-16, drawn to methods of treating insect infestation with compounds affecting ion channel activity.

Group V, claim(s) 17-19, drawn to DNA encoding ion channels, vectors and transformed host cells..

The inventions listed as Groups I and II do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: compounds which affect transcriptional activity genes are structurally different from those which bind to ion channels. Multiple products do not share a special technical feature and thus are not part of a single inventive concept.
The inventions listed as Groups I and III and I and IV do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: compound identification, in vivo treatment of patients and agricultural treatments are different, multiple methods of use and do not share a special technical feature. They do not constitute a single inventive concept. The inventions listed as Groups I and V do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: compounds which bind to ion channels are structurally unrelated to a gene which encodes an ion channel. Multiple products do not share a special technical feature and thus are not part of a single inventive concept.